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August 30, 1999

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13. ABSTRACT *(Maximum 200*

This grant proposes to study the effect of fatty acid synthesis inhibition in human breast cancer xenografts using C75, a novel inhibitor of fatty acid synthesis. C75 has now been shown to have significant anti-tumor activity against the MCF7 human breast cancer xenograft without apparent toxicity to normal proliferating cells. Studies of the mechanisms of cancer cell death from C75 have implicated high levels of malonyl-CoA as the trigger of apoptosis induced by fatty acid synthase inhibition. This is a novel mechanism for generation of apoptosis which may act directly at the mitochondria and will only be operative in cancer cells with high levels of fatty acid synthase. Given that fatty acid synthase is expressed at high levels in many common human cancers and their precursor lesions, this therapeutic strategy may have significant impact on future cancer treatment.

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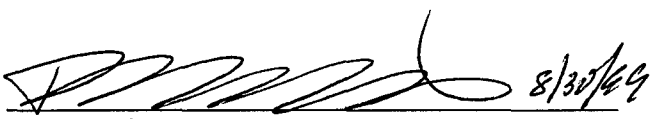

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5. INTRODUCTION: This grant proposed to study the effect of fatty acid synthesis inhibition in human breast cancer xenografts using C75, a novel inhibitor of fatty acid synthesis. We also proposed to study the mechanism of cell death by C75 and the effect of dietary fatty acids on this model. The purpose of this study is to demonstrate that the fatty acid synthesis is a novel pathway for breast cancer therapy development. Since no effective fatty acid synthesis inhibitors exist, we are utilizing C75 which we have developed as the first synthetic, chemically stable inhibitor of mammalian fatty acid synthase. In addition to testing the effects of C75 against breast cancer xenografts, we are also studying the mechanism of action of cell death by C75 in human cancer cells. During the last three years, we have made significant progress toward these goals and have made fundamental observations concerning the mechanism of cancer cell death from fatty acid synthesis inhibition. We now understand that inhibition of fatty acid synthase kills cancer cells through the generation of superphysiological levels of malonyl-CoA which is selectively toxic to cancer cells.

6. BODY:

1. ***Fatty acid synthesis inhibition kills human breast cancer cells through the generation of toxic levels of malonyl-CoA (See preprint in Appendix).***

As I reported one year ago, recent data from Dr. Ellen Pizer showed that pharmacological inhibition of mammalian fatty acid synthase activity lead to inhibition of DNA replication within about 90 minutes of drug application. While generating a great deal of interest, the question of how inhibition of fatty acid synthase triggered this phenomenon remained unknown.

During the last 12 months, we have made a significant breakthrough linking fatty acid synthase inhibition with breast cancer cell apoptosis. In the enclosed preprint of our paper (See Appendix) in review at *Cancer Research*, we demonstrate that inhibition of FAS leads to high levels of malonyl-CoA which occurs within 30 minutes of C75 treatment. These superphysiological levels of malonyl-CoA, ***not low levels of endogenously synthesized fatty acids,*** are responsible for breast cancer cell apoptosis. This finding furthers our understanding of the mechanism of fatty acid synthesis inhibition and cell death. In addition, this is a novel pathway which leads to selective apoptosis of cancer cells. In addition to its role as a substrate for FAS, malonyl-CoA acts at the outer mitochondrial membrane to regulate fatty acid oxidation by inhibition of carnitine palmitoyltransferase 1 (CPT-1). Inhibition of CPT-1 has been shown to sensitize cells to fatty acid induced apoptosis (1); CPT-1 may also interact directly with BCL-2, the anti-apoptosis protein, at the mitochondria (2). We hypothesize that FAS inhibition leads to high levels of malonyl-CoA inhibiting CPT-1 which induces cancer cell apoptosis. Since most proliferating and non-proliferating normal cells do not have high levels of FAS, they will not be affected by this therapeutic strategy.

This paper also demonstrates significant anti-tumor activity of C75 against the MCF7 human breast cancer xenograft. It provides the necessary validation of the preliminary xenograft study documented in the 1998 report. In summary, this paper identifies a novel means to trigger apoptosis in cancer cells through inhibition of fatty acid synthase. and the demonstration of its effectiveness *in vivo.*

Relationship of Studies to the Statement of Work: These studies complete the i.p. model as outlined in Task 5. In addition, it completes analysis of the fatty acid synthesis pathway activity in breast cancer cells *in vitro* and *in vivo* as outlined in Tasks 6 and 7. The discovery of a novel

apoptotic pathway was unanticipated in the original application, but it provides important details of the mechanism of action of C75 and other future FAS inhibitors.

2. Human breast cancer cells have a radically altered distribution of Coenzyme A derivatives in vivo. Since the submission of the paper to *Cancer Research* we have continued to make progress as we can now measure malonyl-CoA levels in xenograft tissues and liver. Figure 1 demonstrates that the MCF-7 human breast cancer xenograft has markedly elevated levels of malonyl-CoA compared to mouse liver. In addition, the distribution of other CoA derivatives are markedly altered. For example, while liver has about 10 fold less malonyl-CoA compared to the xenograft, it has about 10 fold higher levels of acetyl-CoA, and higher levels of other CoA derivatives. These data indicate significant energy metabolism alteration in human cancer cells which merits further investigation. Figure 1B illustrates the quantitative differences in malonyl-CoA levels between the tumor tissue and mouse liver. The methods used for extraction and quantitation of CoA derivatives was the same as in the preprint (See Appendix).

Figure 1A

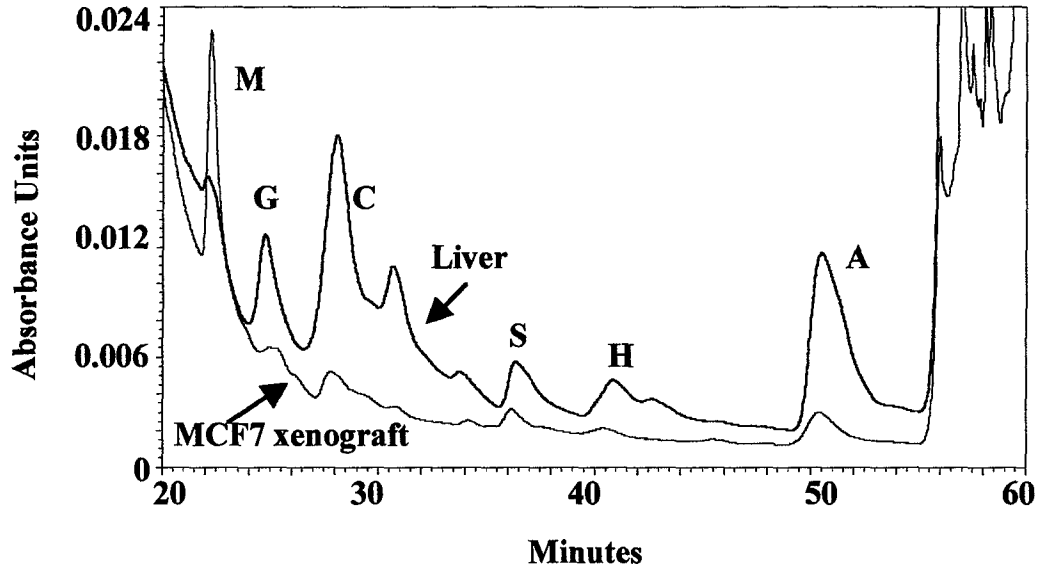


Figure 1B

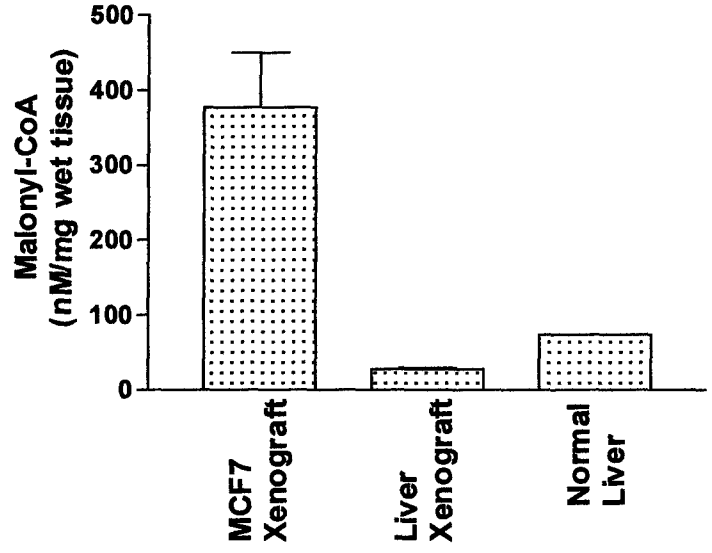


Figure 1A. ***MCF7 xenograft has high steady-state levels of malonyl-CoA.*** HPLC separation of coenzyme-A derivatives from 10% TCA extracts of tumor and liver tissue from the same athymic nude mouse xenografted with MCF7 cells. **M**=malonyl-CoA, **G**=glutaryl-CoA, **C**=free CoA, **S**=succinyl-CoA, **H**=HMG-CoA, **A**=acetyl-CoA. Note the high levels of malonyl-CoA in tumor compared to liver reflecting elevated levels of fatty acid synthesis. All other CoA derivatives are at higher levels in the liver compared to the tumor tissue. **1B.** ***Quantitation of malonyl-CoA in MCF7 xenograft, xenograft liver, and normal liver.*** The MCF7 xenograft has a 10 fold higher level of malonyl-CoA than control liver from the same animal. Normal liver from a female mouse with the estrogen implant without tumor had a higher level of malonyl-CoA than liver from tumor bearing mice. Error bars represent standard error of the mean. Xenograft measurements represent duplicate measurements from the same animal. Only one normal liver sample was tested.

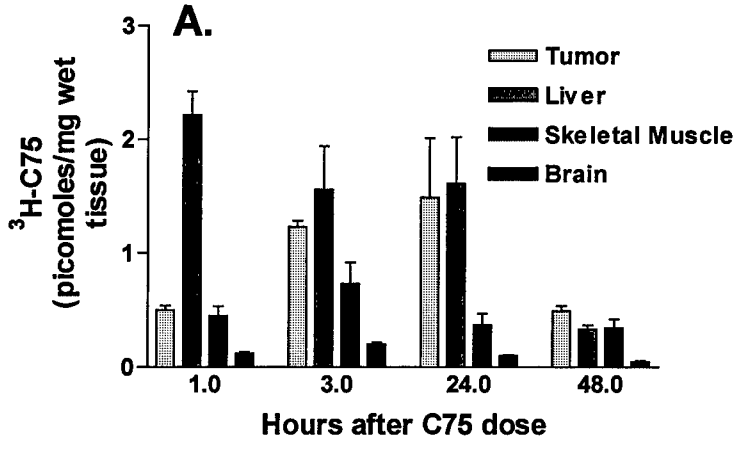
2. Synthesis of [^{3}H-C75].

Dr. Townsend's group has recently synthesized [^{3}H-C75] with a specific activity of 14mCi/mM which is sufficient for *in vitro* and *in vivo* studies. Briefly, the strategy employed to introduce the tritium label involved reduction of a 9 carbon aldehyde with sodium borotritiide to an alcohol with subsequent treatment with PCC (pyridoxine chlorochromate). This labeled aldehyde was then used to synthesize C75 as follows.

Two equivalents of lithiumhexamethyldisilyl amide (LiHMDS) were added to a solution of *p*-methoxybenzyl itaconate dissolved in dry tetrahydrofuran (THF) at -78 °C. After 1 h, an equivalent of [^{3}H-aldehyde] in THF was added at low temperature and stirred for 3-4 h. The reaction was quenched by the addition of cold 6*N* sulfuric acid, and the products were extracted into ether. The organic solution was dried over anhydrous magnesium sulfate and evaporated to a gummy solid, which was dissolved in methylene chloride and treated with trifluoroacetic acid at room temperature for 10-12 h. The products were partitioned into aqueous sodium bicarbonate, reacidified and extracted again into ether. Drying and removal of the solvent as before gave the lactones as a mixture of *trans*- and *cis*-diastereomers as a crystalline solid. These were separated by flash column chromatography on silica gel using ethyl acetate:hexanes:acetic acid 30:70:1 as eluent, and individually crystallized from boiling hexanes.

3. Distribution of [^{3}H-C75] in MCF7 xenograft bearing nude mice.

C75 is widely distributed in tumor and normal tissues, except for the brain. In addition, a large amount of drug remains in the blood which may represent binding to albumin. Figure 2 shows the quantitation of [^{3}H-C75] in the MCF7 xenograft and normal tissues. C75 targets rapidly to liver as expected from i.p. administration. Over 24 hours, tumor C75 levels approximate those of liver. All levels fall dramatically after 48 hours which may represent renal excretion or peripheral metabolism. Importantly, C75 does not reach the brain in significant quantities indicating that it does not easily pass the "blood-brain barrier".



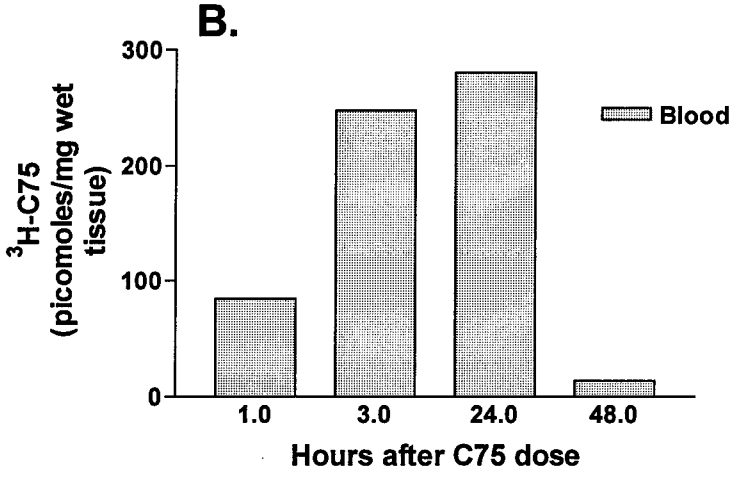


Figure 2A and B. ***Distribution of [^{3}H-C75] in the MCF7 xenograft and normal tissues.*** Athymic nude mice with subcutaneous MCF7 xenografts were treated with 0.6 mg of [^{3}H-C75] i.p. in 200 ul of RPMI vehicle. At the indicated times, animals were sacrificed and 1-2 mg of tissues and 1 μl of blood were removed and counted for ^{3}H. Note the rapid rise in blood and liver levels except for brain. Tumor levels remain elevated above liver after 48 h. Error bars represent standard error of the mean.

Relationship of Studies to the Statement of Work: The synthesis of [^{3}H-C75] and its tissue distribution fulfill tasks 1-3. These data provide substantial support to the C75 xenograft treatment studies demonstrating that labeled drug targets tumor.

4. [^{3}H-C75] binds to FAS in human breast cancer cells in vitro.

In the 1998 report, we demonstrated that C75 inhibits purified mammalian FAS *in vitro*. We now have evidence that C75 binds specifically to FAS in whole cells. 1 x10^5 SKBR3 human breast cancer cells were incubated with 5 μg/ml [^{3}H-C75] for 4 h, washed thrice with PBS, and analyzed by SDS-PAGE and fluorography. Figure 3 shows that [^{3}H-C75] labeled a band at

approximately 260 kDa corresponding to fatty acid synthase as identified by Western blot. Immunoprecipitation of [^{3}H-C75] treated cells with anti-FAS antibodies is in progress. These preliminary data demonstrate that C75 interacts with FAS in whole cells. Furthermore, the interaction of C75 with FAS is consistent with tight binding or a covalent interaction since it survives SDS-PAGE treatment.

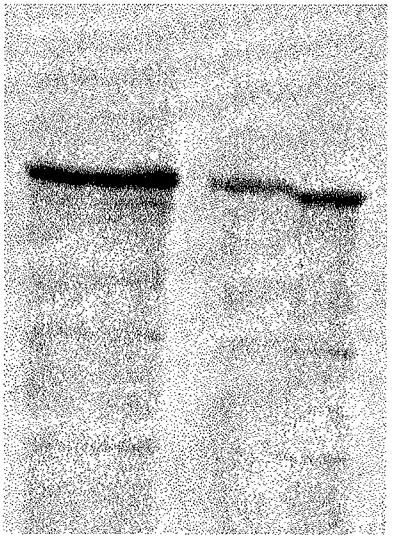


Figure 3 *[^{3}H-C75] binds to an ~260 kDa protein consistent with FAS.* Two samples of 1x10^5 SKBR3 cells each are labeled with [^{3}H-C75] for 4 h, run a 5% SDS-PAGE gel and visualized with flurography. Note the intense band corresponding to the location of FAS on Western blot.

5. C75 induced elevation of malonyl-CoA levels may lead to mitochondrial depolarization. Since C75 induces high levels of malonyl-CoA which inhibit CPT-1 at the outer mitochondrial membrane, we hypothesized that the high levels of malonyl-CoA may lead to mitochondrial depolarization. Respiring mitochondria in healthy cells establish an electrochemical gradient across their membrane called Δpsi_m. Disruption of Δpsi_m has been shown to be one of the first intracellular changes following the onset of apoptosis.

DePsipher (5,5',6,6', tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyaniniodide), a lipophilic cation, aggregates to form an orange-red fluorescent compound during membrane polarization. If the mitochondrial Δpsi_m is perturbed, the dye reverts to a green monomeric form. Figure 4 shows MCF7 cells after a 30 minute exposure to cerulenin. Note the dramatic loss of orange-red fluorescence which is occurring within the time frame of superphysiological accumulation of malonyl-CoA. These preliminary data suggest that malonyl-CoA induces depolarization of Δpsi_m leading to apoptosis.

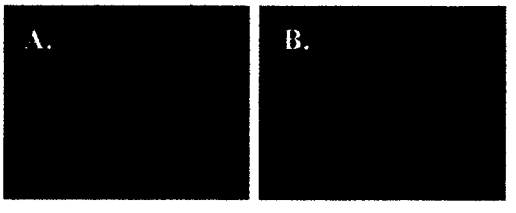


Figure 4 *C75 induces mitochondrial depolarization in SKBR3 cells.* **(A)** SKBR3 cells treated with 5 μg/ml cerulenin for 30 min. Note the significant reduction in red fluorescence which indicates a loss of Δpsi_m compared to **(B)** control cells.

6. C-75 induced weight loss is due to inanition and may represent appetite suppression.

In the last report we detailed a series of studies analyzing the C75 associated weight loss, the only significant C75 toxicity thus far identified. A formal collaboration has been established with Dr M. Daniel Lane, a member of the National Academy of Science and Professor of Biological Chemistry, and Dr. Gabriele Ronnett, Associate Professor of Neuroscience, to further investigate this phenomenon. Further studies have clearly shown that C75 alters feeding habits of mice by reduction of appetite (data not shown).

7. RESEARCH ACCOMPLISHMENTS:

1) Identification of malonyl-CoA as a novel trigger to induce apoptosis in breast cancer cells.
2) Successful treatment of the MCF7 xenograft with C75.
3) Identification of radically altered CoA derivatives in human breast cancer cells *in vivo*.
4) Synthesis of [^{3}H-C75].
5) Demonstration that C75 targets human breast cancer *in vivo*.
6) C75 binds to FAS in intact cells.
7) C75 induces reversible altered feeding behavior in mice leading to weight loss.

8. REPORTABLE OUTCOMES:

Manuscripts:

1. Kuhajda FP, Pizer ES, Mani NS, Pinn ML, Han WF, Chrest FJ, Townsend CA. Synthesis and anti-tumor activity of a novel inhibitor of fatty acid synthase. Proceedings of the American Association of Cancer Research 40: 121, 1999.
2. Parrish NM, Kuhajda FP, Heine HS, Bishai WR, Dick JD. Antimycobacterial activity of cerulenin and its effects on lipid biosynthesis. J. Antimicrobial Chemotherapy, in press,1999.
3. Kuhajda FP. Fatty acid synthase and human cancer: new perspectives on its role in tumor biology. Nutrition (in press).
4. Pizer ES, Thupari J, Han WF, Pinn ML, Chrest FJ, Frehywot GJ, Townsend CA, Kuhajda FP. Malonyl-Coenzyme-A is a Potential Mediator of Cytotoxicity Induced by Fatty Acid Synthase Inhibition in Human Breast Cancer Cells and Xenografts. Submitted to Cancer Research.
5. Loftus TM, Townsend CA, Lane MD, Kuhajda FP. Weight loss mediated by fatty acid synthase inhibition. A link between feeding behavior and fatty acid metabolism. Manuscript in preparation for Science.

Presentations:

Kuhajda FP, Pizer ES, Mani NS, Pinn ML, Han WF, Chrest FJ, Townsend CA. Synthesis and anti-tumor activity of a novel inhibitor of fatty acid synthase. 1999 Annual Meeting of the American Association of Cancer Research, 1999.

Patents:

Inhibition of Fatty Acid Synthase as a Means to Reduce Weight and/or Adipocyte Mass in Humans, Mammals and other Species Capable of Fat Storage"

Granted U.S.application filed November 28, 1996; formal issuance should occur in 1999.

There is a significant patent estate owned by the Johns Hopkins University surrounding diagnostic and therapeutic applications of this technology.

Other grant applications:

We are applying for an NIH RO1 grant for October 1, 1999 to fund further studies on the mechanism of action of FAS inhibition and apoptosis.

In the Spring of 2000, we plan to submit a program project grant with the Departments of Chemistry and Neuroscience to study the biology of feeding behavior and metabolism of FAS inhibition as it applies to weight loss and reduction of adipocyte mass.

9. CONCLUSIONS

Thus far we have demonstrated that FAS inhibition using C75 has a significant anti-tumor effect on the MCF7 xenograft. Radiolabeled C75 has enabled us to directly study the interaction of C75 with FAS and begin pharmacokinetic analysis of C75 in the xenograft. Through studies of C75 mechanism of action we have discovered a novel biochemical pathway leading to apoptosis involving malonyl-CoA. There is now a potential link between FAS inhibition, high levels of malonyl-CoA, CPT-1 inhibition, and the mitochondria to target apoptosis to cancer cells. Further analysis of coenzyme-A derivatives in the MCF7 xenograft and liver has found striking differences indicating profound metabolic abnormalities in cancer which could lead to new target pathways.

Thus, we believe that the medical application of this technology will be significant. C75, the first chemically stable FAS inhibitor has significant anti-tumor activity in breast cancer xenografts without toxicity to proliferating cells. Since many human solid tumors express high levels of FAS and undergo high levels of fatty acid synthesis, this strategy may lead to treatments affecting many common tumors. The high levels of FAS found in *in situ* breast cancer raises the possibility of treatment of precancerous lesions in the future. Since this technology targets a novel pathway, FAS inhibitors may enhance the cytotoxicity of conventional chemotherapy without increasing toxicity to proliferating cells. Finally, these studies seek to expand our fundamental knowledge of metabolism in human breast cancer which may yet provide new insights for therapeutic strategies.

10. REFERENCES.

1) Paumen MB *et al.* Inhibition of carnitine palmitoyltransferase 1 augments sphingolipid synthesis and palmitate-induced apoptosis. J. Biol. Chem. 272, 3324-3329, (1997).

2) Paumen MB *et al.* Direct interaction of the mitochondrial membrane protein carnitine palmitoyltransferase 1 with BCL-2. Biochem. Biophys. Res Com. 231, 523-525 (1997).

Malonyl-Coenzyme-A is a Potential Mediator of Cytotoxicity Induced by Fatty Acid Synthase Inhibition in Human Breast Cancer Cells and Xenografts

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Running title: Malonyl-CoA as a Potential Mediator of Cytotoxicity

Key Words: fatty acid synthase, malonyl-coenzyme A, chemotherapy, apoptosis

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Abstract

A biologically aggressive subset of human breast cancers and other malignancies is characterized by elevated fatty acid synthase (FAS) enzyme expression, elevated fatty acid synthesis and selective sensitivity to pharmacologic inhibition of FAS activity by cerulenin or the novel compound, C75. In this study, inhibition of fatty acid synthesis at the physiologically regulated step of carboxylation of acetyl-CoA to malonyl-CoA by 5-(tetradecyloxy)-2-furoic acid (TOFA) was not cytotoxic to breast cancer cells in clonogenic assays. FAS inhibitors induced a rapid increase in intracellular malonyl-CoA to several fold above control levels, while TOFA reduced intracellular malonyl-CoA by 60%. Simultaneous exposure of breast cancer cells to TOFA and an FAS inhibitor resulted in significantly reduced cytotoxicity and apoptosis. Subcutaneous xenografts of MCF7 breast cancer cells in nude mice treated with C75 showed fatty acid synthesis inhibition, apoptosis, and inhibition of tumor growth to less than one eighth of control volumes, without comparable toxicity in normal tissues. The data suggest that differences in intermediary metabolism render tumor cells susceptible to toxic fluxes in malonyl-CoA, both *in vitro* and *in vivo*.

Introduction

A number of studies have demonstrated surprisingly high levels of fatty acid synthase expression (FAS, E.C. 2.3.1.85) in virulent human breast cancer (1, 2), as well as other cancers (3, 4). FAS expression has also been identified in intraductal and lobular *in situ* breast carcinoma; lesions associated with increased risk for the development of infiltrating breast cancer (5). FAS is the principal synthetic enzyme of fatty acid synthesis (FA synthesis) which catalyzes the NADPH dependent condensation of malonyl-CoA and acetyl-CoA to produce predominantly the 16-carbon saturated free fatty acid, palmitate (6). *Ex vivo* measurements in tumor tissue have revealed high levels of both FAS and FA synthesis indicating that the entire genetic program is highly active consisting of some 25 enzymes from hexokinase to FAS (3). Cultured human cancer cells treated with inhibitors of FAS, including the fungal product, cerulenin, and the novel compound, C75, demonstrated a rapid decline in FA synthesis, with subsequent reduction of DNA synthesis and cell cycle arrest, culminating in apoptosis (7, 8). These findings suggested a vital biochemical link between FA synthesis and cancer cell growth. Importantly, these effects occurred despite the presence of exogenous fatty acids in the culture medium derived from fetal bovine serum. While it has been possible to rescue the cytotoxic effect of cerulenin on certain cells in fatty acid-free culture conditions by the addition of exogenous palmitate, most cancer cells were not rescued from FA synthesis inhibition by the pathway endproduct (data not shown) (9). Thus, it has been unresolved whether the cytotoxic effect of FA synthesis inhibition on most cancer cells resulted from end product starvation, or from some other biochemical mechanism. If fatty acid starvation mediated the cytotoxic effects of cerulenin and C75, then any other FA synthesis inhibitor of similar potency should produce similar effects. To test this idea, we

compared the effects on cancer cells of inhibition of acetyl-CoA carboxylase (ACC, E.C. 6.4.1.2), the rate limiting enzyme of fatty acid synthesis, with the effects of FAS inhibitors.

Figure 1A outlines the portion of the FA synthesis pathway containing the target enzymes of the inhibitors used in this study. TOFA (5-(tetradecyloxy)-2-furoic acid) is an allosteric inhibitor of acetyl-CoA carboxylase (ACC, E.C. 6.4.1.2), blocking the carboxylation of acetyl-CoA to malonyl-CoA. Once esterified to coenzyme-A, TOFA-CoA allosterically inhibits ACC with a mechanism similar to long chain acyl-CoA's, the physiological end-product inhibitors of ACC (10). Both cerulenin (11) and C75 (8) are inhibitors of FAS, preventing the condensation of malonyl-CoA and acetyl-CoA into fatty acids. Cerulenin is a suicide inhibitor, forming a covalent adduct with FAS (12), while C75 is likely a slow-binding inhibitor (13). We now report that using TOFA we achieved FA synthesis inhibition in human breast cancer cell lines comparable to inhibition by cerulenin or C75. Surprisingly, however, TOFA was essentially non-toxic to human breast cancer cells. These data suggest that fatty acid starvation is not a major source of cytotoxicity to cancer cells in serum supplemented culture. Rather, high levels of the substrate, malonyl-CoA, resulting specifically from inhibition of FAS, may mediate cytotoxicity of cerulenin and C75.

Materials and Methods

Fatty acid synthesis inhibitors. Cerulenin was obtained from Sigma. C75 and TOFA were synthesized in the laboratory of C.A. Townsend in the Department of Chemistry, at the Johns Hopkins University.

Cell lines, culture conditions, metabolic labeling, and clonogenic assays. The human breast cancer cell lines, SKBR3 and MCF7 were maintained in RPMI with 10% fetal bovine serum. Cells were screened periodically for *Mycoplasma* contamination (Gen-probe). All inhibitors were added as stock 5 mg/ml solutions in DMSO. For fatty acid synthesis activity determinations, $5x10^4$ cells/well in 24 well plates were pulse labeled with [U-^{14}C]-acetate after exposure to drug, and lipids were extracted and quantified as described previously (8). For MCF7 cells, pathway activity was determined after 2 hours of inhibitor exposure. SKBR3 cells demonstrated slower response to FAS inhibitors, possibly because of their extremely high FAS content, so pathway activity was determined after 6 hours of inhibitor exposure. For clonogenic assays, $4x10^5$ cells were plated in 25 cm^2 flasks with inhibitors added for 6 hours in concentrations listed. Equal numbers of treated cells and controls were plated in 60mm dishes. Clones were stained and counted after 7 to 10 days.

Flow-cytometric quantitation of apoptosis. Apoptosis was measured by multiparameter flow cytometry using a FACStar^{Plus} flow cytometer equipped with argon and krypton lasers (Becton Dickinson). Apoptosis was quantified using merocyanine 540 staining (Sigma), which detects altered plasma membrane phospholipid packing that occurs early in apoptosis, added directly to cells from culture (8, 14). In some experiments, chromatin conformational changes of apoptosis were simultaneously measured as decreased staining with LDS-751 (Exciton) (15). Merocyanine 540 [10μg/ml] was added as a 1 mg/ml stock in water. Cells were stained with LDS-751 at a final concentration of 100nM from a 1mM stock in DMSO. The merocyanine 540-positive cells were marked by an increase in red fluorescence, collected at 575 +/- 20 nm, 0.5 to 2 logs over merocyanine 540-negative cells. Similarly, the LDS-751 dim cells demonstrated a reduction in

fluorescence of 0.5 to 1.5 logs relative to normal cells, collected at 660 nm with a DF20 band pass filter. Data were collected and analyzed using CellQuest software (Becton Dickinson). In these experiments, all LDS-751 dim cells were merocyanine 540 bright, however a population of merocyanine 540 bright cells were detected that were not yet LDS-751 dim. All merocyanine 540 bright cells were classified as apoptotic.

Measurement of malonyl-CoA. Malonyl-CoA levels were measured in MCF-7 cells using the HPLC method of Corkey, *et al* (16). Briefly, 2.5 x 10^5 cells/well in 24 well plates were subjected to 1.2 ml of 10% TCA at 4° C after various drug treatments. The pellet mass was recorded and the supernatant was washed 6 times with 1.2 ml of ether and reduced to dryness using vacuum centrifugation at 25° C. Coenzyme-A esters were separated and quantitated using reversed phase HPLC on a 5 μ Supelco C18 column with a Waters HPLC system running Millenium^{32} software monitoring 254nm as the maximum absorbance for coenzyme-A. The following gradients and buffers were utilized: Buffer A: 0.1 M potassium phosphate, pH 5.0, Buffer B: 0.1 M potassium phosphate, pH 5.0, with 40% acetonitrile. Following a 20 min. isocratic run with 92% A, 8% B at 0.4 ml/min, flow was increased to 0.8 ml/min over one minute whereupon a linear gradient to 10% B was run until 24 min. then held at 10% B until 50 min. where a linear gradient was run to 100% B at 55 min., completing at 60 min. The following coenzyme-A esters (Sigma) were run as standards: malonyl-CoA, acetyl-CoA, glutathione-CoA, succinyl-CoA, HMG-CoA, and free CoA. Samples and standards were dissolved in 50 μl of buffer A. Coenzyme-A esters eluted sequentially as follows: malonyl-CoA, glutathione-CoA, free CoA, succinyl-CoA, HMG-CoA, and acetyl-CoA. Quantitation of coenzyme-A esters was performed by the Millenium^{32} software.

Xenograft Studies. Subcutaneous flank xenografts of the human breast cancer cell line, MCF-7 in nu/nu female mice (Harlan) were used to study the anti-tumor effects of C75 *in vivo*. All animal experiments complied with institutional animal care guidelines. All mice received a 90-day slow-release subcutaneous estrogen pellet (Innovative Research) in the anterior flank 7 days before tumor inoculation. 10^7 MCF7 cells were xenografted from culture in DMEM supplemented with 10% FBS and insulin 10 μg/ml. Treatment began when measurable tumors developed about 10 days after inoculation. Eleven mice (divided among two separate experiments of 5 and 6 mice each) were treated intraperitoneally with weekly doses of C75 at 30 mg/kg in 0.1 ml RPMI. Dosing was based on a single dose LD_{10} determination of 40 mg/kg in BALB/c mice; 30 mg/kg has been well tolerated in outbred nude mice. Eleven control mice (divided in the same way as the treatment groups) received RPMI alone. Tumor volume was measured with calipers in three dimensions. Experiment was terminated when controls reached the surrogate endpoint. In a parallel experiment to determine fatty acid synthesis activity in treated and control tumors, a group of MCF-7 xenografted mice were treated with C75 or vehicle at above doses and sacrificed after 3 hours. Tumor and liver tissue were *ex vivo* labeled with [U^{14}-C], lipids were extracted and counted as described (3). In an additional parallel experiment to histologically examine treated and control tumors, 6 C75 treated and 6 vehicle control mice were sacrificed 6 hours after treatment. Tumor and normal tissues were fixed in neutral-buffered formalin, processed for routine histology, and immunohistochemistry for FAS was performed.

FAS Immunohistochemistry. Immunohistochemistry for FAS was performed on the MCF-7 xenografts using a mouse monoclonal anti-FAS antibody (1) at 1:2000 on the Dako Immunostainer using the LSAB2 detection kit.

Results and Discussion

TOFA, Cerulenin, and C75 all inhibited fatty acid synthesis in human breast cancer cells, but showed differential cytotoxicity. In standard pulse labeling experiments in which breast cancer cell lines, SKBR3 and MCF7 were labeled for 2 hours after exposure to FA synthesis inhibitors, TOFA, C75, and cerulenin all inhibited [U^{14}C-acetate] incorporation into lipids to a similar extent (Figure 1B and D). In numerous similar experiments (not shown), TOFA maximally inhibited FA synthesis in the 1 to 5μg/ml dose range in all cell lines tested, and cerulenin and C75 maximally inhibited FA synthesis in the range of 10μg/ml. Although all inhibitors reduced FA synthesis to a similar degree, TOFA was non-toxic or stimulatory to the cancer cell growth in the dose range for ACC inhibition, as measured by clonogenic assays, while cerulenin and C75 were significantly cytotoxic in the dose range for FAS inhibition (Figure 1C and E). The profound difference between the cytotoxic effects of ACC and FAS inhibition demonstrated that the acute reduction of fatty acid production *per se*, was not the major source of cell injury after FAS inhibition. Alternatively, these data suggested that cytotoxicity resulted from a biochemical effect of FAS inhibition that was not shared by ACC inhibition.

Malonyl-CoA levels were markedly increased with FAS inhibition and reduced by TOFA. The most obvious difference in the expected results of inhibiting these two enzymes was that

malonyl-CoA levels should fall after ACC inhibition, but should increase after FAS inhibition. Although not previously investigated in eukaryotes, recent data in *E. coli* have demonstrated elevated levels of malonyl-CoA resulting from exposure to cerulenin (17).

Direct measurement of coenzyme-A derivatives in MCF-7 cells by reversed phase HPLC of acid soluble extracts from drug treated cells confirmed that both cerulenin and C75 caused a rapid increase in malonyl-CoA levels while TOFA reduced malonyl-CoA levels. Figure 2A is a representative chromatograph demonstrating the separation and identification of coenzyme-A derivatives important in cellular metabolism. Malonyl-CoA is the first of these to elute, with a column retention time of 19-22 minutes. The overlay of chromatographs in Figure 2B shows that cerulenin treatment lead to a marked increase in malonyl-CoA over the control while TOFA caused a significant reduction. The chemical identity of the malonyl-CoA was independently confirmed by spiking samples with standards (not shown). Analysis of multiple experiments in Figure 2C demonstrated that following a 1 hour exposure to cerulenin or C75 at 10 μg/ml, malonyl-CoA levels increased by 930% and 370% respectively, over controls, while TOFA treatment (20 μg/ml) led to a 60% reduction of malonyl-CoA levels. The concentration of TOFA required for maximal reduction of malonyl-CoA levels was 4 fold higher than the dose for pathway inhibition in Figure 1B and D. However, optimal cultures for extraction of CoA derivatives had 5 fold higher cell density than the cultures used in the other biochemical and viability assays presented. The remarkable increase in malonyl-CoA after FAS inhibition can be attributed in part to the release of long-chain fatty acyl-CoA inhibition of ACC leading to an increase in ACC activity (Figure 1A). Moreover, the cerulenin-induced increase in malonyl-CoA levels occurred within 30 minutes of treatment (930 +/-15% increase over control, not

shown), within the time frame of FA synthesis inhibition, and well before the onset of DNA synthesis inhibition or early apoptotic events (8). Thus, high levels of malonyl-CoA were a characteristic effect of FAS inhibitors and temporally preceded the other cellular responses, including apoptosis.

Inhibition of ACC rescued breast cancer cells from FAS inhibition. If the elevated levels of malonyl-CoA resulting from FAS inhibition were responsible for cytotoxicity, then it should be possible to rescue cells from FAS inhibition by reducing malonyl-CoA accumulation with TOFA. Co-administration of TOFA and cerulenin to SKBR3 cells (Figure 3A) abrogated the cytotoxic effect of cerulenin alone in clonogenic assays. In MCF7 cells (Figure 3C), TOFA produced a modest rescue of both cerulenin and C75 under similar experimental conditions. Representative flow cytometric analyses of SKBR3 cells (Figure 3B) and MCF7 (Figure 3D) substantiated these findings, since TOFA rescued cells from cerulenin induced apoptosis. These experiments also confirmed the differential cytotoxicity between TOFA (<5% increase in apoptosis; no reduction in clonogenicity) compared to cerulenin (>85% apoptosis; 70% reduction in clonogenicity). Taken together, these studies suggest that high malonyl-CoA levels may play a role in the cytotoxic effect of FAS inhibitors on cancer cells.

***In vivo* inhibition of FAS lead to reduced tumor growth.** Previous studies have demonstrated local efficacy of cerulenin against a human cancer xenograft (18), but were limited by the failure of cerulenin to act systemically. The similar responses of breast cancer cells to cerulenin and C75 *in vitro* suggested that C75 might be effective *in vivo* against xenografted breast cancer cells. To determine if the effects of FAS inhibition seen *in vitro* would translate to an *in vivo* setting

requiring systemic activity, we tested C75 against subcutaneous MCF-7 xenografts in athymic nude mice, to quantitate effects on FA synthesis and the growth of established solid tumor.

Fatty acid synthesis pathway activity in tissues of xenografted mice was determined by *ex vivo* pulse labeling with [U^{14}C]-acetate. The tumor xenografts had 10-fold higher FA synthesis activity than liver, highlighting the difference in pathway activity between benign and malignant tissues (Figure 4A). FAS expression in the MCF-7 xenograft paralleled the high level of FA synthesis activity (Figure 4B). Intraperitoneal injections of C75 at 30 mg/kg reduced fatty acid synthesis in *ex vivo* labeled liver by 76% and in the MCF-7 xenografts by 70% within 3 hours (Figure 4A). These changes in FA synthesis preceded histological evidence of cytotoxicity in the xenograft, which became evident 6 hours after treatment (Figures 4 C and 4D). The C75 treated xenografts showed numerous apoptotic bodies throughout the tumor tissue, which were not seen in vehicle treated tumors. Histological analysis of liver and other host tissues following C75 treatment showed no evidence of any short or long term toxicity (not shown).

Weekly intraperitoneal C75 treatment retarded the growth of established subcutaneous MCF-7 tumors compared to vehicle controls, demonstrating a systemic anti-tumor effect (Figure 4E). After 32 days of weekly treatments, there was a greater than eight-fold difference in tumor growth in the treatment group compared to vehicle controls. Similar to cerulenin, transient reversible weight loss was the only toxicity noted (18).

The systemic pharmacologic activity of C75 provided the first analysis of the outcome of systemic FAS inhibitor treatment. The significant anti-tumor effect of C75 on a human breast

cancer xenograft in the setting of physiological levels of ambient fatty acids was similar to the *in vitro* result in serum supplemented culture, and was consistent with a cytotoxic mechanism independent of fatty acid starvation. Furthermore, the result suggested that malonyl-CoA accumulation may not be a significant problem in normal tissues, possibly because FA synthesis pathway activity is normally low, even in lipogenic organs such as the liver. It is of further interest that, while malonyl-CoA was the predominant low molecular weight CoA congugate detected in breast cancer cells in these experiments, other studies have reported predominantly succinyl-CoA and acetyl-CoA in cultured hepatocytes (16). Differences in CoA derivative profiles may be indicative of larger differences in energy metabolism between cancer cells and hepatocytes.

The identification of malonyl-CoA as a potential mediator of cytotoxicity, possibly via induction of apoptosis in cancer cells, while unanticipated, was not surprising given its pivotal role in cellular metabolism. In addition to its function as a substrate for fatty acid synthesis, malonyl-CoA regulates fatty acid oxidation by inhibiting carnitine palmitoyltransferase I (CPT-1) at the outer mitochondrial membrane (19). Physiologically, the elevated levels of malonyl-CoA occurring during FA synthesis reduce fatty acid oxidation to prevent a futile cycle of simultaneous fatty acid synthesis and degradation. During starvation or feeding with high fat diets, fat synthesis ceases, malonyl-CoA levels fall, and fatty acids enter the mitochondrion for energy production. Malonyl-CoA is thus a crucial regulatory metabolic intermediate in cellular energy metabolism. How superphysiologic levels of malonyl-CoA may lead to apoptosis is not yet known, however, CPT-1, which is regulated by malonyl-CoA, has been shown to interact directly with Bcl-2 at the mitochondrial membrane (20). This convergence suggests that high

levels of malonyl-CoA may either induce apoptosis directly, or may alter mitochondrial metabolism to increase susceptibility to apoptosis from other signals. Thus, further investigation of malonyl-CoA and CoA metabolism in cancer cells may yield new insights into cancer cell metabolism and selective susceptibility to anti-metabolite therapy.

Bibliography

1. Alo, P. L., Visca, P., Marci, A., Mangoni, A., Botti, C., and Di Tondo, U. Expression of fatty acid synthase (FAS0 as a predictor of recurrence in stage I breast carcinoma patients., Cancer. *77:* 474-482, 1996.
2. Jensen, V., Ladekarl, M., Holm-Nielsen, P., Melsen, F., and Soerensen, F. B. The prognostic value of oncogenic antigen 519 (OA-519) expression and proliferative activity detected by antibody MIB-1 in node-negative breast cancer., Journal of Pathology. *176:* 343-352, 1995.
3. Rashid, A., Pizer, E. S., Moga, M., Milgraum, L. Z., Zahurak, M., Pasternack, G. R., Kuhajda, F. P., and Hamilton, S. R. Elevated expression of fatty acid synthase and fatty acid synthetic activity in colorectal neoplasia., American Journal of Pathology. *150:* 201-208, 1997.
4. Pizer, E., Lax, S., Kuhajda, F., Pasternack, G., and Kurman, R. Fatty acid synthase expression in endometrial carcinoma: correlation with cell proliferation and hormone receptors., Cancer. *83:* 528-537, 1998.
5. Milgraum, L. Z., Witters, L. A., Pasternack, G. R., and Kuhajda, F. P. Enzymes of the fatty acid synthesis pathway are highly expressed in in situ breast carcinoma., Clinical Cancer Research. *3:* 2115-2120, 1997.

6. Wakil, S. Fatty acid synthase, a proficient multifunctional enzyme., Biochemistry. *28:* 4523-4530, 1989.

7. Pizer, E. S., Jackisch, C., Wood, F. D., Pasternack, G. R., Davidson, N. E., and Kuhajda, F. Inhibition of fatty acid synthesis induces programmed cell death in human breast cancer cells., Cancer Research. *56:* 2745-2747, 1996.

8. Pizer, E. S., Chrest, F. J., DiGiuseppe, J. A., and Han, W. F. Pharmacological inhibitors of mammalian fatty acid synthase suppress DNA replication and induce apoptosis in tumor cell lines., Cancer Research. *58:* 4611-4615, 1998.

9. Pizer, E. S., Wood, F. D., Pasternack, G. R., and Kuhajda, F. P. Fatty acid synthase (FAS): A target for cytotoxic antimetabolities in HL60 promyelocytic leukemia cells., Cancer Research. *1996:* 745-751, 1996.

10. Halvorson, D. L. and McCune, S. A. Inhibition of fatty acid synthesis in isolated adipocytes by 5-(tetradecyloxy)-2-furoic acid., Lipids. *19:* 851-856, 1984.

11. Funabashi, H., Kawaguchi, A., Tomoda, H., Omura, S., Okuda, S., and Iwasaki, S. Binding site of cerulenin in fatty acid synthetase., J. Biochem. *105:* 751-755, 1989.

12. Moche, M., Schneider, G., Edwards, P., Dehesh, K., and Lindqvist, Y. Structure of the complex between the antibiotic cerulenin and its target, beta-ketoacyl carrier protein synthase., J Biol Chem. *274:* 6031-6034, 1999.

13. Kuhajda FP, Pizer ES, Mani NS, Pinn ML, Han WF, Chrest FJ, and CA, T. Synthesis and anti-tumor activity of a novel inhibitor of fatty acid synthase., Proceeding of the American Association for Cancer Research. *40:* 121, 1999.

14. Mower, D. A., Peckham, D. W., Illera, V. A., Fishbaugh, J. K., Stunz, L. L., and Ashman, R. F. Decreased membrane phospholipid packing and decreased cell size

precede DNA cleavage in mature mouse B cell apoptosis, J Immunol. *152:* 4832-4842, 1994.

15. Frey, T. Nucleic acid dyes for detection of apoptosis in live cells., Cytometry. *21:* 265-274, 1995.

16. Corkey, B. E. Analysis of acyl-coenzyme A esters in biological samples, Methods in Enzymology. *166:* 55-70, 1988.

17. Chohnan, S., Furukawa, H., Fujio, T., Nishihara, H., and Takamura, Y. Changes in the size and composition of intracellular pools of nonesterified coenzyme A and coenzyme A thioesters in aerobic and facultatively anaerobic bacteria., Applied and Environmental Microbiology. *63:* 553-560, 1997.

18. Pizer, E. S., Wood, F. D., Heine, H. S., Romantsev, F. R., Pasternack, G. R., and Kuhajda, F. P. Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer., Cancer Research. *56:* 1189-1193, 1996.

19. Witters, L. and Kemp, B. Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase., J. Biol. Chem. *267:* 2864-2867, 1992.

20. Paumen, M. B., Ishisa, Y., Han, H., Muramatsu, M., Eguchi, Y., Tsujimoto, Y., and Honjo, T. Direct interaction of the mitochondrial membrane protein carnitine palmitoyltransferase I with Bcl-2, Biochem Biophys Res Commun. *231:* 523-525, 1997.

Figure Legends

Figure 1. Inhibitors of the fatty acid synthesis pathway. (A) Schematic representation of the fatty acid synthesis pathway showing the specificity of cerulenin and C75 for FAS and of TOFA for ACC. The three FA synthesis inhibitors reduced FA synthesis activity (incorporation of [$U^{14}C$]-acetate into extractable lipids) by comparable amounts in SKBR3 breast carcinoma cells **(B)** and in MCF7 breast carcinoma cells **(D)**. The cytotoxic activity of the three FA synthesis inhibitors, was determined by clonogenic assay in the dose range for FA synthesis inhibition. Six hour exposure to cerulenin or C75 reduced the clonogenic fraction of SKBR3 breast carcinoma cells **(C)** and MCF7 breast carcinoma cells **(E)**, while TOFA did not.

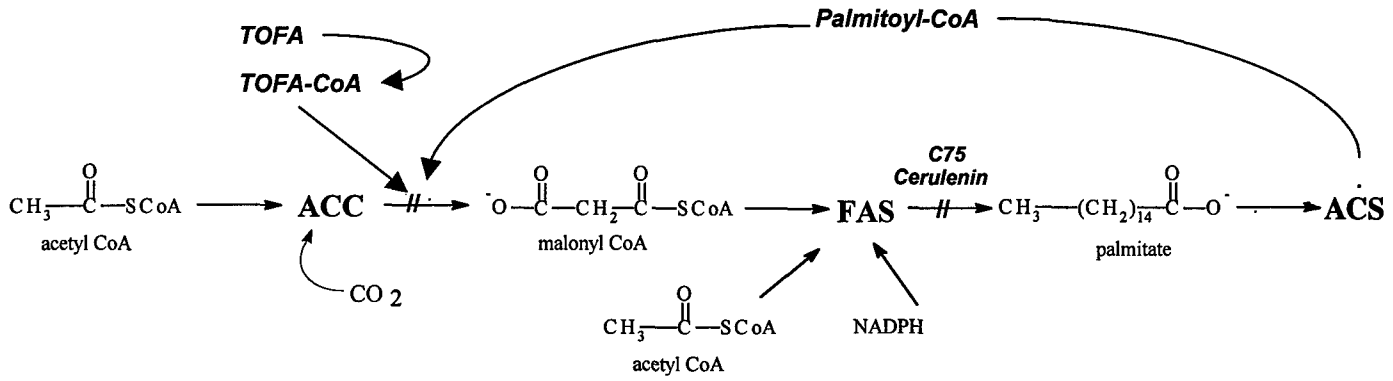
Figure 2. Effects of the FA synthesis inhibitors on malonyl-CoA levels. Chemical standards **(A)**, And acid soluble extracts of MCF7 breast carcinoma cells **(B)** were resolved by reversed phase HPLC for quantitation of malonyl-CoA. Changes in intracellular malonyl-CoA content after exposure to the three FA synthesis inhibitors are shown in **(C)**. Assays were performed in triplicate.

Figure 3. ACC inhibition rescued the cytotoxic effects of FAS inhibition in breast cancer cells. (A) Pretreatment with TOFA rescued SKBR3 cells from cerulenin cytotoxicity as determined by clonogenic assays. **(C)** Similarly, TOFA reduced both cerulenin and C75 cytotoxicity in MCF7 cells. Using merocyanine 540 staining as an indicator of apoptosis, TOFA rescued both SKBR3 cells **(B)** and MCF7 cells **(D)** from cerulenin cytotoxicity.

Figure 4. Selective cytotoxicity of the FAS inhibitor, C75, to MCF7 breast carcinoma flank xenografts in nude mice. (A) Xenografted tumor had high FA synthesis activity relative to liver, determined by *ex vivo* metabolic labeling. A standard intraperitoneal dose of C75 [30mg/kg] inhibited FA synthesis in both liver and tumor by 76% and 70% respectively at three hours. **(B)** FAS expression was elevated in the xenografted tumor in parallel with FA synthesis, determined by immunohistochemistry. **(C and D)** A standard intraperitoneal dose of C75 [30mg/kg] produced histologic evidence of widespread apoptosis in the xenografted tumor at six hours **(D)** which was not evident in vehicle treated animals **(C). (E)** Weekly treatment with intraperitoneal C75 [30mg/kg] inhibited the growth of established MCF7 xenografts, resulting in a greater than eight-fold difference in mean tumor growth between vehicle and drug treated tumors after 32 days.

Figure 1.

A.



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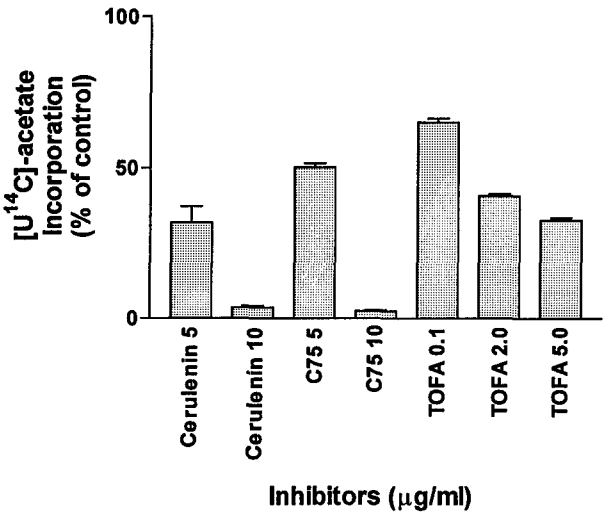

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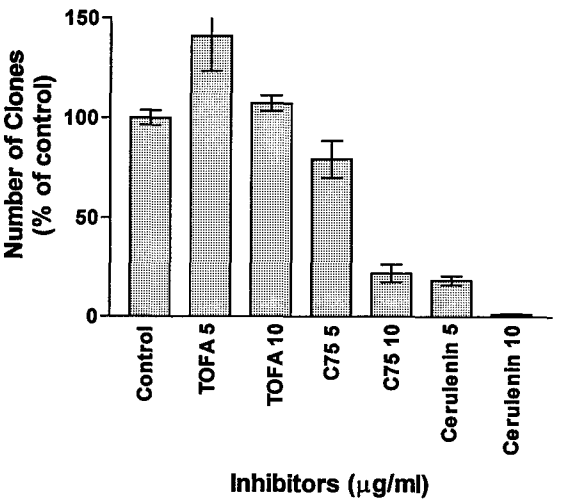

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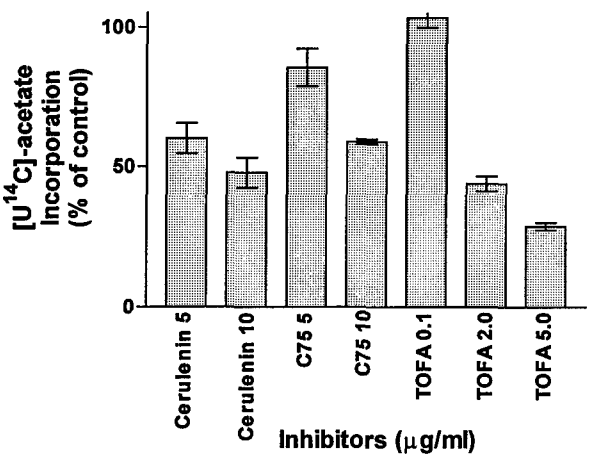


E.

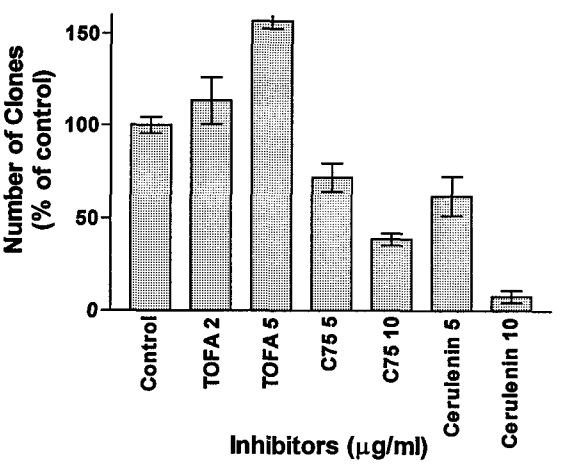
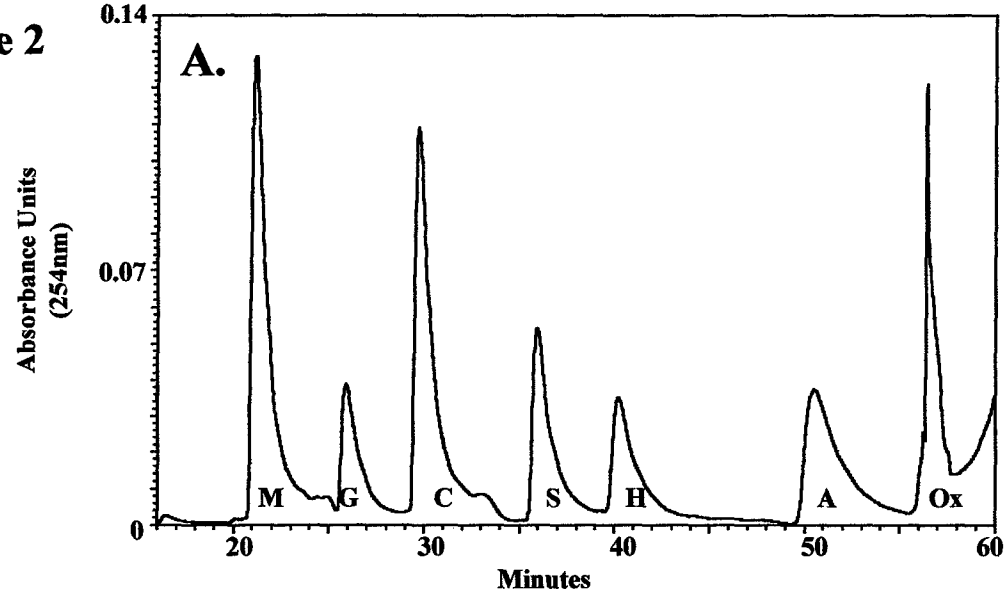
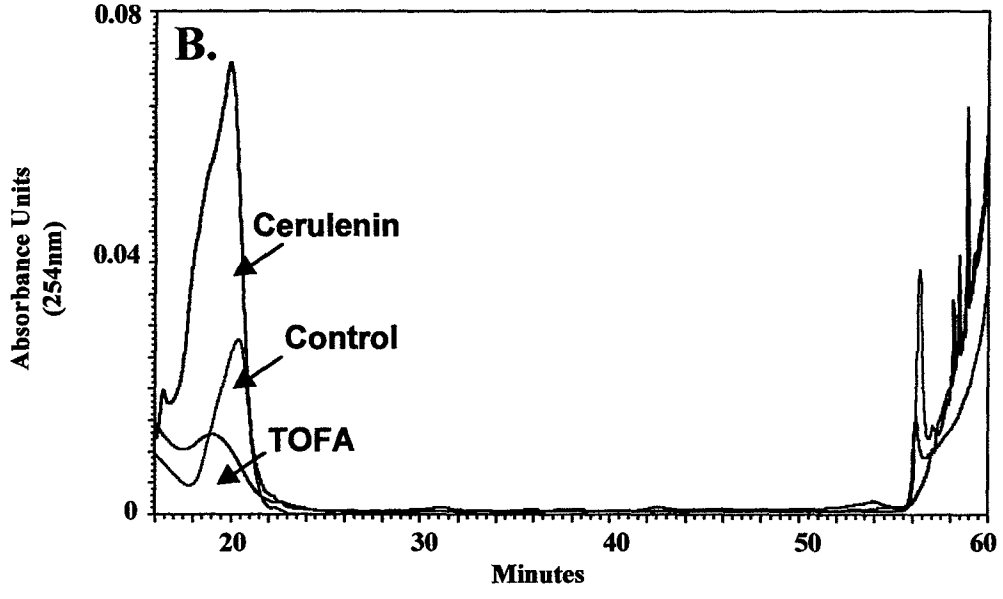


Figure 2


A.
0.14
0.07
0
Absorbance Units (254nm)
M
G
C
S
H
A
Ox
20
30
40
50
60
Minutes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Cerulenin
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TOFA
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50
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Minutes

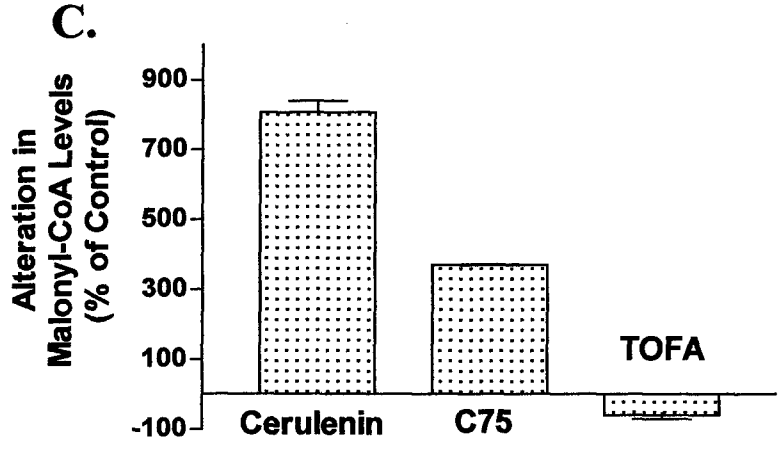

C.
Alteration in Malonyl-CoA Levels (% of Control)
900
700
500
300
100
-100
Cerulenin
C75
TOFA

Figure 3.

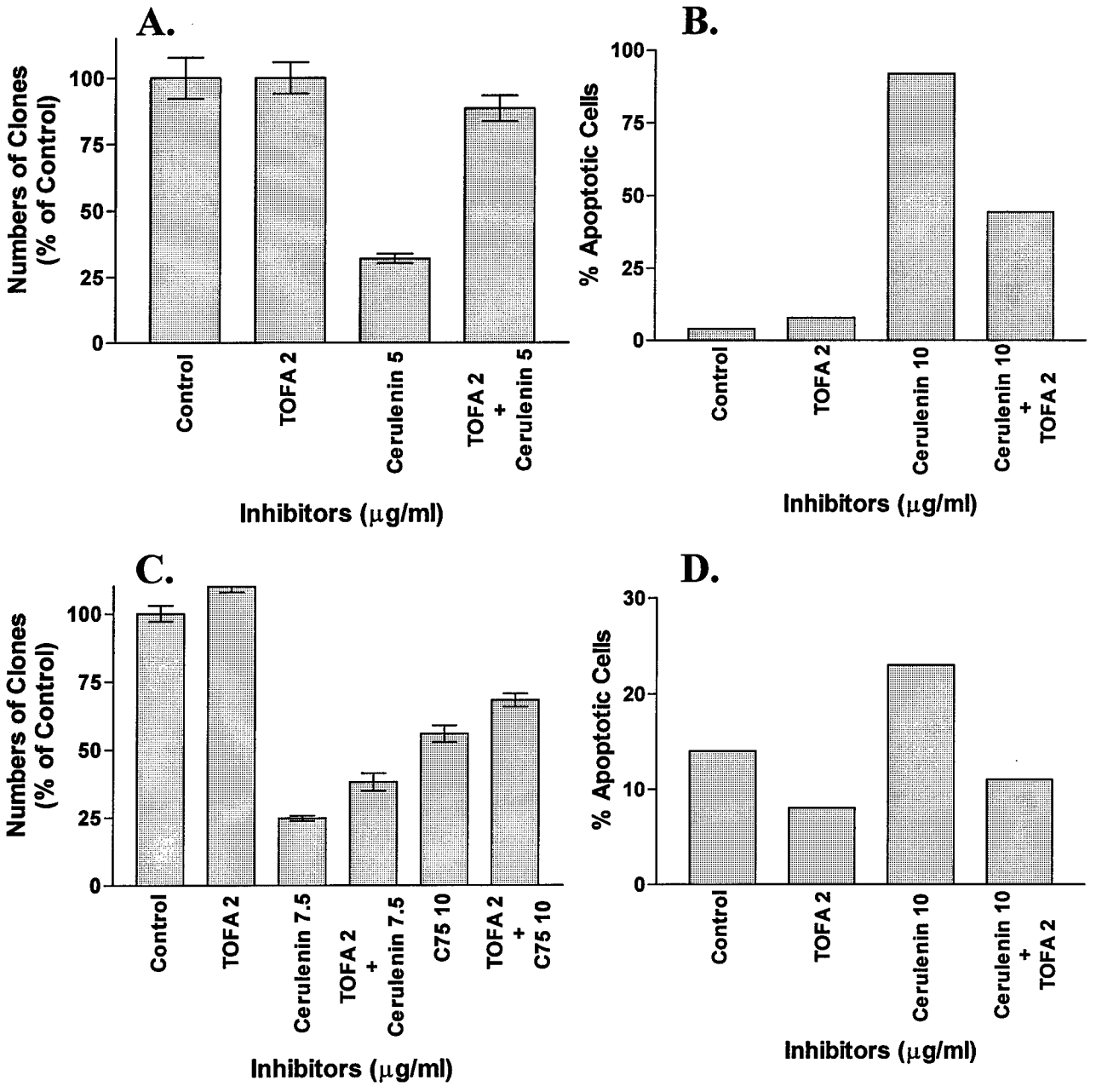
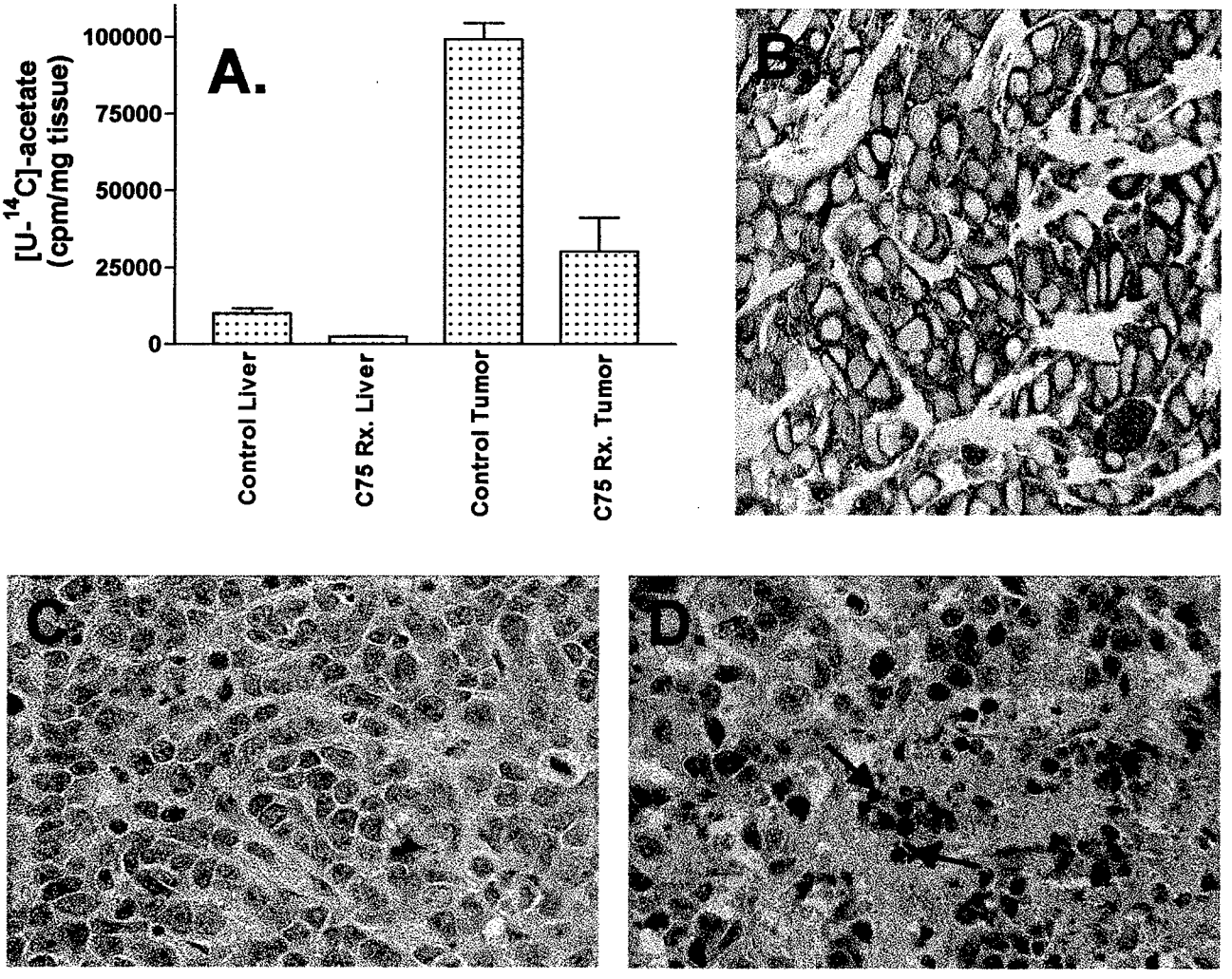

A.
Numbers of Clones (% of Control)
100
75
50
25
0
Control
TOFA 2
Cerulenin 5
TOFA 2 + Cerulenin 5
Inhibitors (μg/ml)
B.
% Apoptotic Cells
100
75
50
25
0
Control
TOFA 2
Cerulenin 10
Cerulenin 10 + TOFA 2
Inhibitors (μg/ml)
C.
Numbers of Clones (% of Control)
100
75
50
25
0
Control
TOFA 2
Cerulenin 7.5
TOFA 2 + Cerulenin 7.5
C75 10
TOFA 2 + C75 10
Inhibitors (μg/ml)
D.
% Apoptotic Cells
30
20
10
0
Control
TOFA 2
Cerulenin 10
Cerulenin 10 + TOFA 2
Inhibitors (μg/ml)

Figure 4


A.
[U-14C]-acetate (cpm/mg tissue)
100000
75000
50000
25000
0
Control Liver
C75 Rx. Liver
Control Tumor
C75 Rx. Tumor
B.
C.
D.
E.
Tumor Volume (% increase over pre-treatment volume)
40
30
20
10
0
C75
Control
0
5
10
15
20
25
30
35
40
Time (days)